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Active Heymann nephritis in complement component C6 deficient rats

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Active Heymann nephritis in complement component C6 deficient rats. The mechanisms of renal injury that result in proteinuria in active Heymann nephritis (AHN) remain unclear, though data suggest that in analogy of the passive form of the disease the membrane attack complex C5b-9 may be involved. AHN was induced in an inbred strain of PVG/c$^-$ rats that are totally deficient in the C6 component of complement and are unable to form the lytic C5b-9 complex, as well as in non-complement deficient PVG/c$^+$ rats that are immunologic identical to the deficient strain. In both groups of animals comparably high titers of anti-Fx1A autoantibodies were found after three weeks and persisted at 40 weeks. Proteinuria was also similar in both groups, and was first evident at six weeks. High levels of urinary protein, ranging from 200 mg/24 hr to 500 mg/24 hr, were found after 10 weeks and persisted up to one year. Renal biopsy findings at various times post-immunization were identical in both groups, including immunofluoresence staining for Ig and C3 deposits, and also EM findings of subepithelial electron-dense deposits were not different. The injection of heterologous rabbit complement, that partially and temporarily restored the CH_{50} activity in PVG/c$^-$ rats did not alter or hasten the disease. Long-term follow-up showed that all rats in both groups continued to have severe proteinuria and that most animals died between 8 to 12 months after disease induction, without renal impairment. EM findings in serial biopsies demonstrated that the growth of the subepithelial deposits as measured by surface area occurred between weeks 4 and 12. A positive correlation ($r = 0.94$) between the size of the deposits and the level of proteinuria was found. These studies demonstrate that the membrane attack complex of complement does not play a major role in AHN. The relationship of the size of the immune deposits to the level of proteinuria suggests that the growth of the immune deposits on itself initiate secondary mechanisms that damage the permselective characteristics of the glomerular membrane.

Active Heymann nephritis (AHN) was first reported in 1959 as an experimental model of human membranous nephropathy that was induced by immunization of rats with homologous kidney homogenate in complete Freund adjuvant [1]. Later studies showed that the responsible nephritogenic antigens present in the homogenate were of renal tubular origin [2–4]. The disease is characterized by the appearance of autoantibodies and the formation of subepithelial electron-dense deposits after two to three weeks. Frank proteinuria is seen within two months of immunization. In contrast to passive Heymann nephritis (PHN) that is rapidly obtained by administration of heterologous antibody to the homogenate Fx1A, AHN is a true autoimmune disease with a slow evolution [5].

The discovery that the pathogenesis of Heymann nephritis (HN) was not caused by circulating immune complexes but by *in situ* binding of antibodies to intrinsic epithelial antigens and local formation of deposits was an important step in unraveling the disease mechanisms of this experimental model [6–8]. This finding showed that glomerular membrane damage was not caused by passage of immune complexes. Salant and colleagues later hypothesized that the terminal components of complement unified in the membrane attack complex (MAC) C5b-9 were responsible for the early damage of the glomerular membrane in PHN [9]. Since then, several reports have highlighted the role of the C5b-9 complex in membranous nephropathy [reviewed in 5, 10, 11].

Due to the variability of the disease and the inability to decomplement the rats over a long time period it has never been shown that proteinuria is also complement mediated in AHN [5]. Nevertheless, indirect evidence is available that complement also plays a role in AHN [12] and even in other forms of human glomerulonephritis [13, 14].

We have recently discovered a colony of PVG/c rats totally deficient in component C6 and unable to form a functional C5b-9 complex as measured by its inability to lyse sensitized sheep RBC [15]. These rats were designated PVG/c$^-$. All other colonies of PVG/c rats showed normal complement activity. One of these, designated PVG/c$^+$, was found to be immunologically identical to the PVG/c$^-$ rat [15].

To investigate whether the absence of functional C5b-9 complex was of significance for the development and evolution of AHN we induced the disease in PVG/c$^-$ and PVG/c$^+$ rats.

Methods

Animal models

Inbred male and female PVG/c$^+$ and C6 deficient PVG/c$^-$ rats eight to nine weeks old and weighting between 160 and 220 g were used in all experiments. PVG/c$^+$ rats were obtained from B&K Universal Ltd. (Hull, UK). PVG/c$^-$ rats were bred in our own animal facilities. The characteristics of these rats have been described elsewhere [15].

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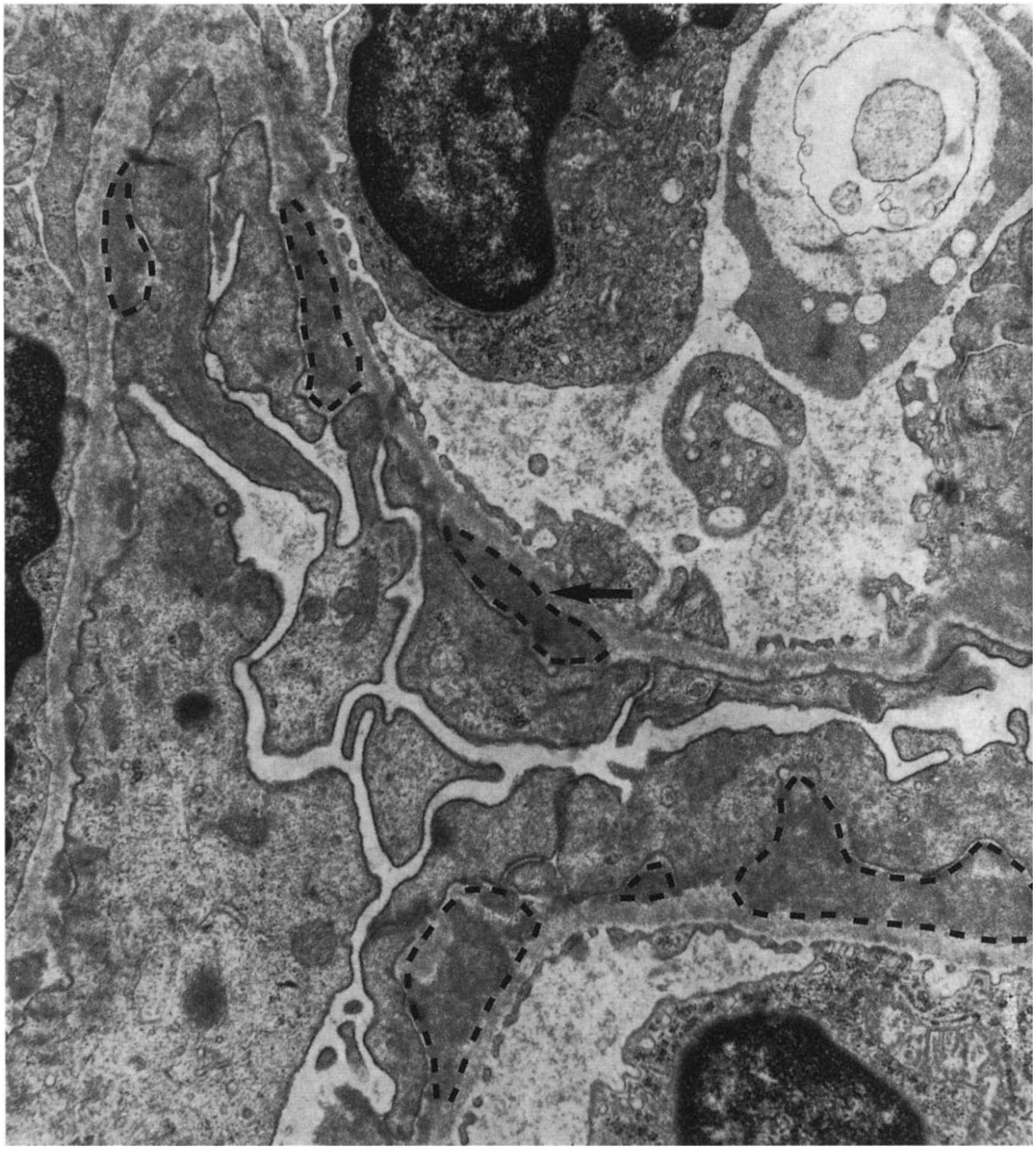

Fig. 1. *Example of measuring the size of immune deposits by measuring the surface area with a planimeter.* Only the three largest deposits of five randomly chosen capillary loops of five randomly chosen glomeruli per biopsy were measured. Magnification was 10,000×. The encircled deposit (arrow) measured 0.47 μm^2.

Rats were maintained on standard chow and water *ad libitum*. During 24-hour urine collections rats were deprived of chow.

AHN was induced by a single injection in the left hind food pad of 15 mg of brush border antigens Fx1A, prepared as described [16] and dissolved in 0.15 ml complete Freund adjuvant containing Mycobacterium tuberculosis HRa37 (Difco, Detroit, MI, USA). No booster vaccination was administered.

Monitoring of active Heymann nephritis

Twenty-four hour urine samples collected in metabolic cages were assayed for protein by the biuret method. Blood was obtained from the lateral vein of the tail of rats anesthetized with ether. Serum was stored at −80°C until further assayed for autoantibody titer, serum creatinine and functional complement.

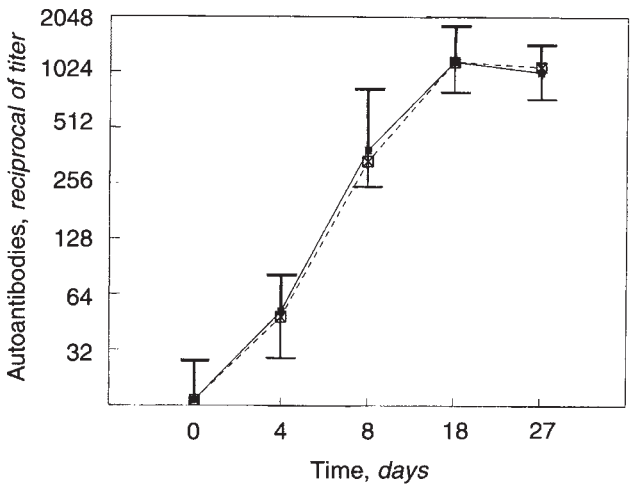

Fig. 2. *Anti-Fx1A autoantibody titers of PVG/c$^+$ (—⊠—) and PVG/c$^-$ (—■—).* The number of animals were ten and nine, respectively. The ordinate represents the reciprocal of the dilutions on a $\log_2$ scale. Values are means ± SD.

Anti-Fx1A autoantibody titers were determined by ELISA as described [17]. Briefly, microtiter plates (NUNC, Roskilde, Denmark) were coated overnight with 1 μg of Fx1A in 0.1 ml PBS. Plates were washed three times with PBST (PBS with 0.05% Tween 20) and blocked with 2% bovine serum albumin (BSA) for one hour at room temperature followed by two washes. Diluted rat sera were added to the plates for one hour at 37°C. A dilution series of strong positive and negative sera were used as references. After three washes the plates were incubated at 37°C with goat anti-rat Ig alkaline phosphatase (Caltag, San Francisco, CA, USA) diluted 1/500 in BSA 2% and washed again three times with PBST and once with carbonate-bicarbonate buffer. Finally, 0.1 ml 0.5% p-nitrophenyl phosphate (Sigma, St. Louis, MO, USA) was added for 15 minutes. The yellow color reaction was read at 405 nm on an ELISA reader. The titer was determined as the reciprocal of the last dilution that differed significantly from negative serum. Arbitrarily, the level of positivity was determined as the mean value of absorbance of the diluted negative serum augmented with two standard deviations.

Serum creatinine was measured with a standard photocolorimetric method on samples previously depleted of lipids by ultracentrifugation at 46,000 × g.

Lytic activity of complement was monitored with the CH50 assay [17, 18]. Briefly, sheep red blood cells (SRBC) were first sensitized with decomplemented rabbit anti-SRBC serum to become activated erythrocytes (EA). Hemolysis was measured by reading the optical density of the supernatants at 541 nm. The reciprocal of the serum dilution that resulted in 50% hemolysis is defined as the CH50 value expressed in units per ml. A control serum containing 52 CH50 units per ml (Sigma) was used as a standard.

Kidney biopsies were taken by a surgical procedure with partial resection of the cortex of the lower pole of the left kidney. Up to three biopsies were taken in the same animal during the follow-up period. Part of the tissue was snap frozen in liquid nitrogen and stored at −80°C for immunofluorescence studies. Another part of the tissue was fixed in glutaraldehyde 4% in PBS for electron microscopy (EM).

Immunofluorescent staining of frozen biopsies was performed with rabbit anti-rat Ig and with FITC conjugated anti-rat C3, both prepared in the laboratory of one of us (M.D.). The intensity of the granular deposits was scored as reported [19]. EM was done to assess immune deposit size at various stages of the disease. The size of the immune deposits was related to the time post-immunization and the level of proteinuria. Photomicrographs with a magnification of 10,000 × were taken of five randomly chosen capillary loops of a particular glomerulus. Of each biopsy five randomly chosen capillary loops were investigated. The surface area of the three largest immune deposits observed in each biopsy was measured with a planimeter that was accurate up to 10 mm^2 (Haff, Pfronten, Germany) or, taken into account the magnification in the microscope and in the photographic print, up to 0.02 μm^2 (Fig. 1). The reasoning behind this strategy was that several investigators have claimed that the C5b-9 complex is demonstrable in the deposits and this complex might have caused a substantial growth of the deposits as its size is several million Daltons. It was not excluded that part of the disease was determined by the size of the deposits. In general, more than thirty deposits were demonstrable in five capillary loops per biopsy. The foregoing methodology is also a quantitative method to measure the size of the deposits. Indeed, to quantitavely determine the size of the deposits from the two-dimensional photographs two questions have to be resolved. First, what is the distribution of the size of the deposits and second how to determine the size of the deposits from the sections seen on the photographs. Starting with the latter, if all deposits have equal size and showed a spherical configuration then it will be clear that the largest two-dimensional deposit will be a section through the equator plane of the deposit. The cross sectional areas of the deposits are related to the cosinus fuction, for example a section at 20° from the equator has a surface area of $\pi(\text{Radius}_{\text{equator}} \cos 20°)^2$. This is more than 88% of the surface area of the equator plane. Thus, any section within the range ± 20° of the equator plane closely represents the size of the deposit. The frequency of random cross sections of the sfere are represented by the sinus function, for example, the probability to find a section between the equator plane and the plane at 20° is given by sinus 20° which is 34%. Using the binomial distribution it can be shown that the probability to find at least 3 out of 30 deposits within the 34% interval is more than 98%. Thus, the mean value of the three largest two-dimensional deposits is a good estimation for the equator plane. The foregoing reasoning is by approximation still valid when a disc-shaped configuration for the deposits is assumed. This configuration with the long axis twice the short axis ressembles much more the real two-dimensional sections. If the size of the deposits is gaussian distributed with a small standard deviation then the foregoing is an accurate determination of the size of the deposits. From three biopsies we measured all two-dimensional deposits and found in all three that, when we ranked the surface areas from each biopsy from small to large, about 50% of the deposits showed a surface area of 75% or more of the largest surface area. Such a distribution ressembles the sinus and cosinus function of above [sinus 30° = 0.5; (cosinus 30°)^2 = 0.75]. This finding implicates that the gaussian distribution must be narrow. With other words all deposits develop in an equal way and reach sizes that do not differ much to each other. Taken together, measuring the three largest two-dimensional deposits out of a series of more than thirty gives a good estimation of the magnitude of the deposits. The method has the advantages

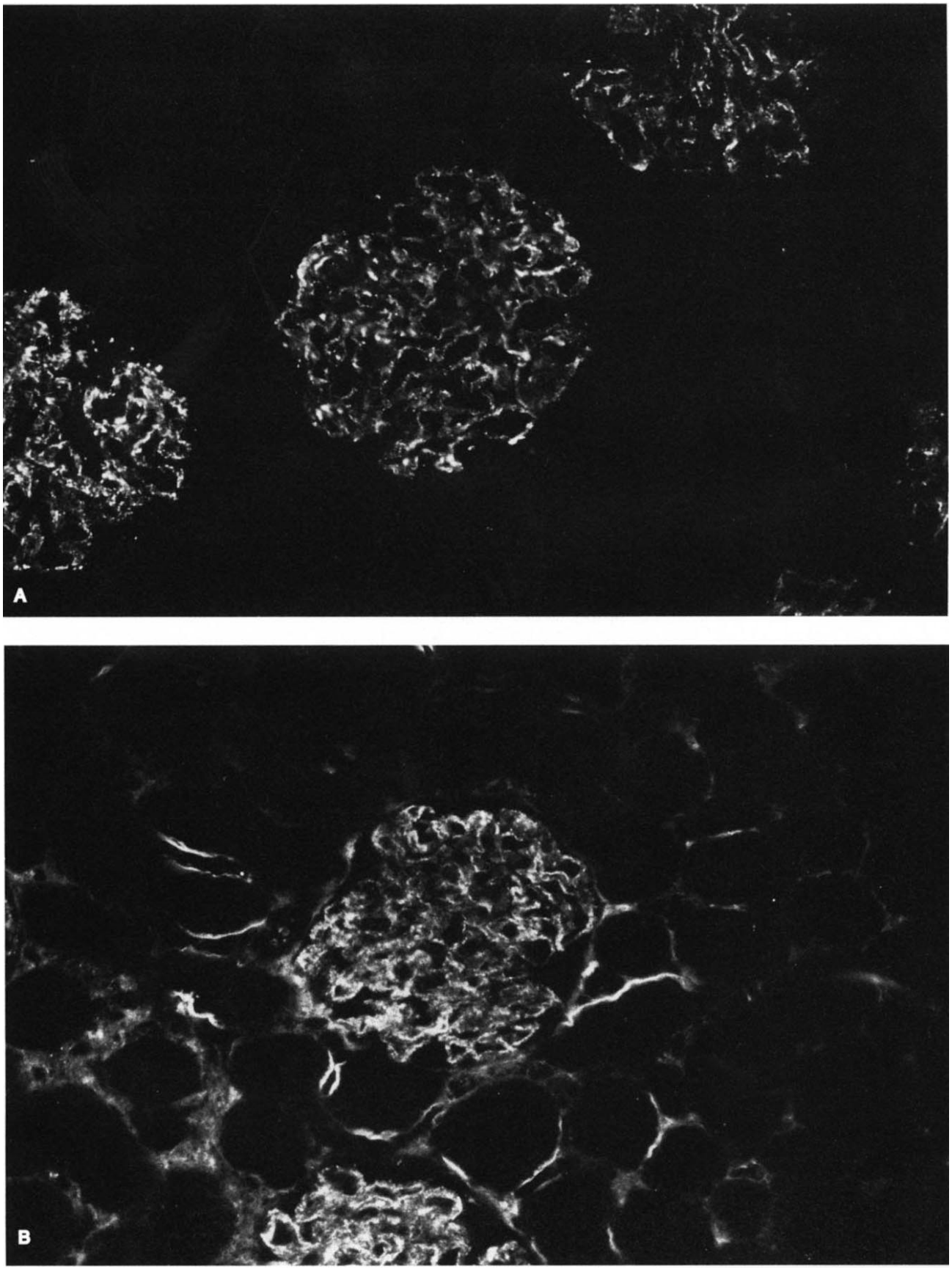

Fig. 3. *Immunofluorescence staining for rat Ig* (**A**) *and rat C3* (**B**) *at four weeks after induction of AHN in a PVG/c⁻ rat.* Staining intensities did not differ to those of non-complement deficient rats.

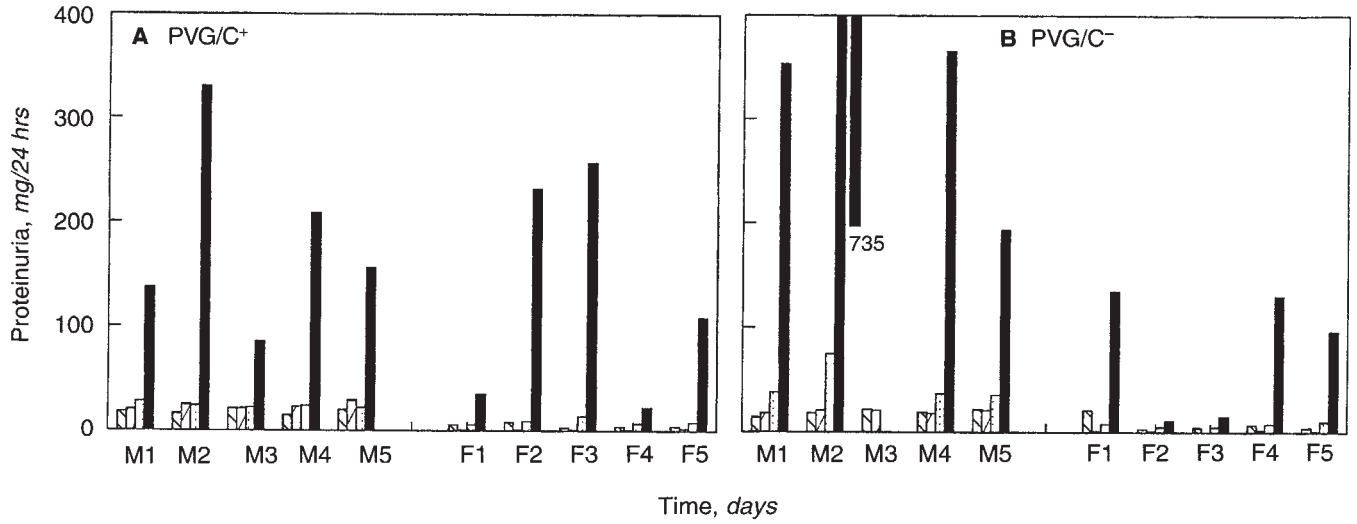

Fig. 4. *Proteinuria of 24 hours of the first ten rats of each group at days (▨) 0, (▨) 28, (▨) 42, and (■) 56.* Male M3 of the PVG/c⁻ group died during surgery at four weeks. Females F2 and F3 of the same group showed 14 and 17 mg per day of proteinuria at 56 days, respectively. This is significantly different to non-induced females (less then 5 mg per day). By week twelve their urinary protein loss had increased to 48 and 86 mg per day, respectively.

Table 1. Evolution of proteinuria and serum creatinine

	Week 4	Week 8	Week 12	Week 20	Week 40
Proteinuria *mg/day*					
PVG/c⁺					
Males	12.2 ± 3.1 (10)	112 ± 54 (10)	231 ± 68 (10)	276 ± 90 (5)	344 ± 102 (3)
Females	2.7 ± 1.6 (10)	44 ± 37 (10)	129 ± 42 (10)	188 ± 68 (5)	174 ± 46 (4)
PVG/c⁻					
Males	14.4 ± 4.9 (10)	214 ± 106 (9)	328 ± 86 (9)	312 ± 72 (5)	412 ± 67 (2)
Females	3.2 ± 1.4 (10)	52 ± 27 (10)	154 ± 54 (10)	235 ± 74 (5)	248 ± 88 (4)
Creatinine *mg/dl*					
PVG/c⁺	0.36 ± 0.04 (20)	0.42 ± 0.05 (20)	0.47 ± 0.08 (20)	0.44 ± 0.07 (10)	0.68 ± 0.09 (7)
PVG/c⁻	0.34 ± 0.05 (20)	0.39 ± 0.05 (19)	0.49 ± 0.08 (19)	0.52 ± 0.10 (10)	0.74 ± 0.12 (6)

Males and females are separately represented for proteinuria. The values are means ± SD. Values between parentheses are number of rats involved. None of the animals at any time point showed a level of serum creatinine of more than 1 mg/dl. At week 12 the first ten rats of each group were eliminated.

Table 2. CH50 in complement reconstituted PVG/c⁻ rats

Rat	Day 1	Day 2	Day 8
1	46	30	8
2	26	22	<5
3	30	20	12
4	42	18	<5
5	22	10	<5
6	30	10	<5

CH50 is expressed in U/ml and represents the reciprocal of the dilution that causes 50% hemolysis of antibody-coated sheep RBC. The CH50 value of normal PVG/c⁺ serum and rabbit serum was measured 84 U/ml and 110 U/ml, respectively. At day 8 most animals showed no detectable CH50 value. At day 15 (not shown), CH50 was demonstrable in none of the six rats.

of being less time consuming and more accurate in absolute values than determining the mean value of all two-dimensional deposits.

Experimental design

AHN was induced in two groups of PVG/c⁺ and PVG/c⁻ rats, each containing ten male and ten female animals. Proteinuria was determined in all animals at regular time intervals. The first ten animals of each group were used to monitor AHN during the first three months, whereafter these animals were sacrificed as repetitive biopsies might have impaired the natural evolution of their renal function. From these rats blood was drawn at regular time intervals and biopsies were taken monthly during the first three months. The other ten rats of each group were used for long-term follow-up and were further monitored from month three until natural death or for one year. During this period repetitive biopsies were taken in some rats.

In a second series of experiments AHN was induced in two groups of animals each containing six male PVG/c⁻ rats. The first group was the control group. The rats of the second group were injected i.v. with 1 ml of heterologous rabbit complement at week three after AHN induction when autoantibodies were present in serum and glomeruli but rats did not yet show proteinuria. The evolution of the two groups of animals was monitored.

Statistics

Statistical calculations were performed using the Mann-Whitney non-paired tests for proteinuria and the Student's *t*-test for serum autoantibody and serum creatinine. Linear regression was

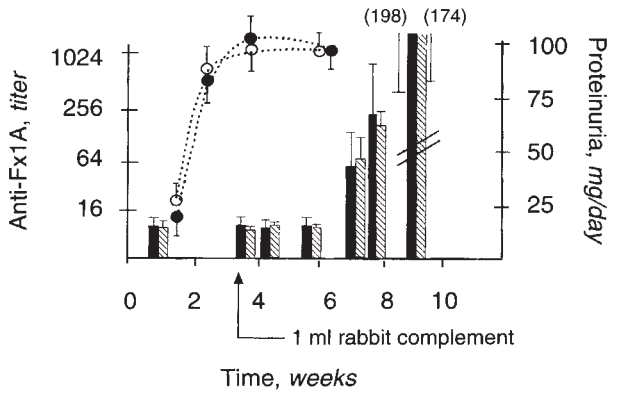

Fig. 5. *Administration of heterologous rabbit complement does not alter the disease evolution in PVG/c⁻ rats.* Autoantibody titers (circles) and proteinuria (bars) are represented for two groups of male PVG/c⁻ rats ($N = 6$) of which one was not reconstituted (closed symbols) and the other group (open symbols) was injected with one ml of fresh rabbit serum. Values are means ± SD. Figures between parenthesis represent mean proteinuria.

done between size of immune deposits and level of proteinuria. $P < 0.05$ was considered a significant difference.

Results

Autoantibody production and immunofluorescent studies

Both PVG/c⁺ and PVG/c⁻ rats rapidly raised anti-Fx1A antibodies as shown in Figure 2. Antibodies started to appear after one week and reached high levels at week three whereafter the titers remained invariably high throughout the lifespan of the rats. No difference was seen in titer between the two rat colonies at any time point.

Similar results were obtained when biopsies were stained for rat Ig and rat C3 (Fig. 3). At week four, fine granular immunofluorescence of equal intensity was observed in both PVG/c⁺ and PVG/c⁻ rats.

Proteinuria

Basal proteinuria in both rat colonies matched each other. Males showed levels between 5 mg/day and 12 mg/day, whereas the basal urinary protein loss in females always was less than 5 mg/day. Unmanipulated rats did not develop disease and did not show an increase in basal proteinuria by aging. After induction of AHN the rats remained free of proteinuria during the first four weeks. At week six several animals showed significant levels of proteinuria and by week eight the majority to these rats became severely proteinuric showing protein losses of more than 100 mg/day. Figure 4 represents the individual values of proteinuria during the first eight weeks of the first ten rats of each group. Though variable levels were measured all rats developed marked proteinuria. No clear difference was seen between PVG/c⁺ and PVG/c⁻ rats. Females tended to develop proteinuria a little later and to a little lesser extent that males (Table 1). Once the rats showed severe proteinuria they continued to have high levels of urinary protein loss during their lifespan. Proteinuria of more than 0.4 g/day, turning the sera of lipids milky, was common. As expected, edema was not observed in these animals [20].

Reconstitution with heterologous complement

Apparently both rat colonies developed proteinuria, but as proteinuria was only determined every two and four weeks PVG/c⁺ rats could have developed the disease a little earlier than the complement deficient ones. Though in this case the mechanism of renal injury could not solely be attributed to the C5b-9 complex, it still might contribute to the development of the disease. Also, regarding homologous restriction of complement [21], where homologous cells are at least partially protected against homologous complement, it was meaningful to reconstitute the complement deficient rats with heterologous rabbit complement at the time anti-Fx1A antibodies were present and yet no significant proteinuria had developed. We reasoned that, if in this case no influence was seen on the evolution of the disease, it would definitely have been shown that the C5b-9 complex played no role in AHN. Two groups of PVG/c⁻ rats were induced with AHN. One of them was injected with 1 ml of rabbit serum at week three, when anti-Fx1A antibodies were present. Hemolytic activity was partially and temporarily reconstituted as shown in Table 2. Nevertheless, the development of proteinuria was not accelerated nor was an increase in the levels of proteinuria observed demonstrating that administration of heterologous complement with reconstitution of hemolytic activity had no influence in AHN (Fig. 5).

Ultrastructural studies

In AHN, proteinuria develops six weeks after induction, whereafter levels of proteinuria of several hundred mg per day are reached within four to six weeks. To answer the question of what happens during this time period sequential biopsies were taken in several rats. In all animals a marked increase of the size of the deposits was observed over time (Fig. 6). Also, large deposits caused detachment of the glomerular epithelial cells and distortion of the slit pore membranes. Simultaneously with the increases of the size of the complexes an increase in proteinuria was found. Figure 7 shows the relationship between the size of the three largest deposits found in the biopsy and proteinuria. Small deposits, present at four weeks after induction, had not caused significant proteinuria. Between a surface area of 0.20 and 1.20 μm^2 of the deposits a fairly linear relationship between size of the deposits and proteinuria was found. These data were obtained from biopsies taken six to twelve weeks after induction. Later biopsies and some biopsies taken at twelve weeks are represented in area 3. A clear linear relationship is no longer present. The maximal size of the deposits seems limited to 2.0 μm^2. After more than twelve weeks neither the size of the deposits nor the proteinuria changed for the individual rat (not shown). Thus, before proteinuria develops, a minimal size of the deposits has to be reached. Further growth over time results in an increase in proteinuria.

Long term follow-up

Table 1 shows the evolution of proteinuria and serum creatinine of PVG/c⁺ and PVG/c⁻ rats. All rats developed comparable levels of proteinuria while no rats developed severe renal insufficiency. The small rise in serum creatinine can at least partially be explained by the repetitive biopsies which caused loss of kidney mass. The survival graph is represented in Figure 8. A major number of rats died between weeks 36 and 52 while non-diseased rats live for more than two years (not shown). The cause of death

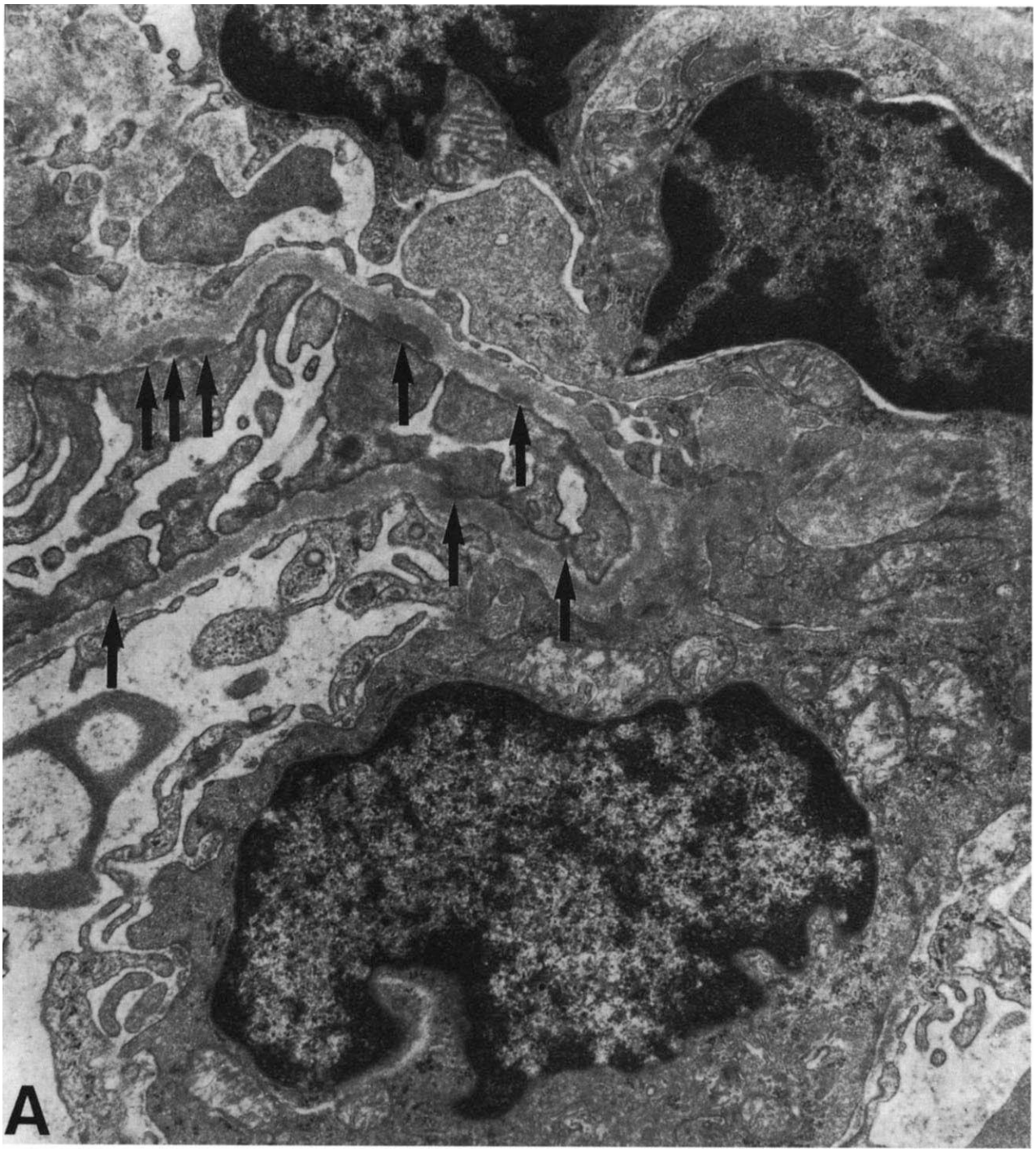

Fig. 6. *EM findings in PVG/c⁻ rat M5 at four (***A***), six (***B***) and eight (***C***) weeks after induction of AHN.* Proteinurias were 12 mg per day, 25 mg per day and 188 mg per day, respectively. Notice the marked increase of the size of the immune deposits and the flattening of the glomerular epithelial cells (arrows). Magnification was 10,000 ×.

is not immediately clear and does not seem to be related to impaired renal function. Though not investigated, thrombotic accidents may be a plausible explanation for sudden death in these nephrotic animals.

However, no difference was observed between deficient and normal rats, which shows that C6 deficiency had no influence on the natural evolution of the nephrotic rats.

Discussion

The mechanisms of renal injury in AHN have not been conclusively resolved. By analogy to PHN it was assumed that complement, and more particularly the MAC, which is composed of the C5b-9, was responsible for mediating glomerular injury that results in proteinuria [10–12]. This contention was supported by

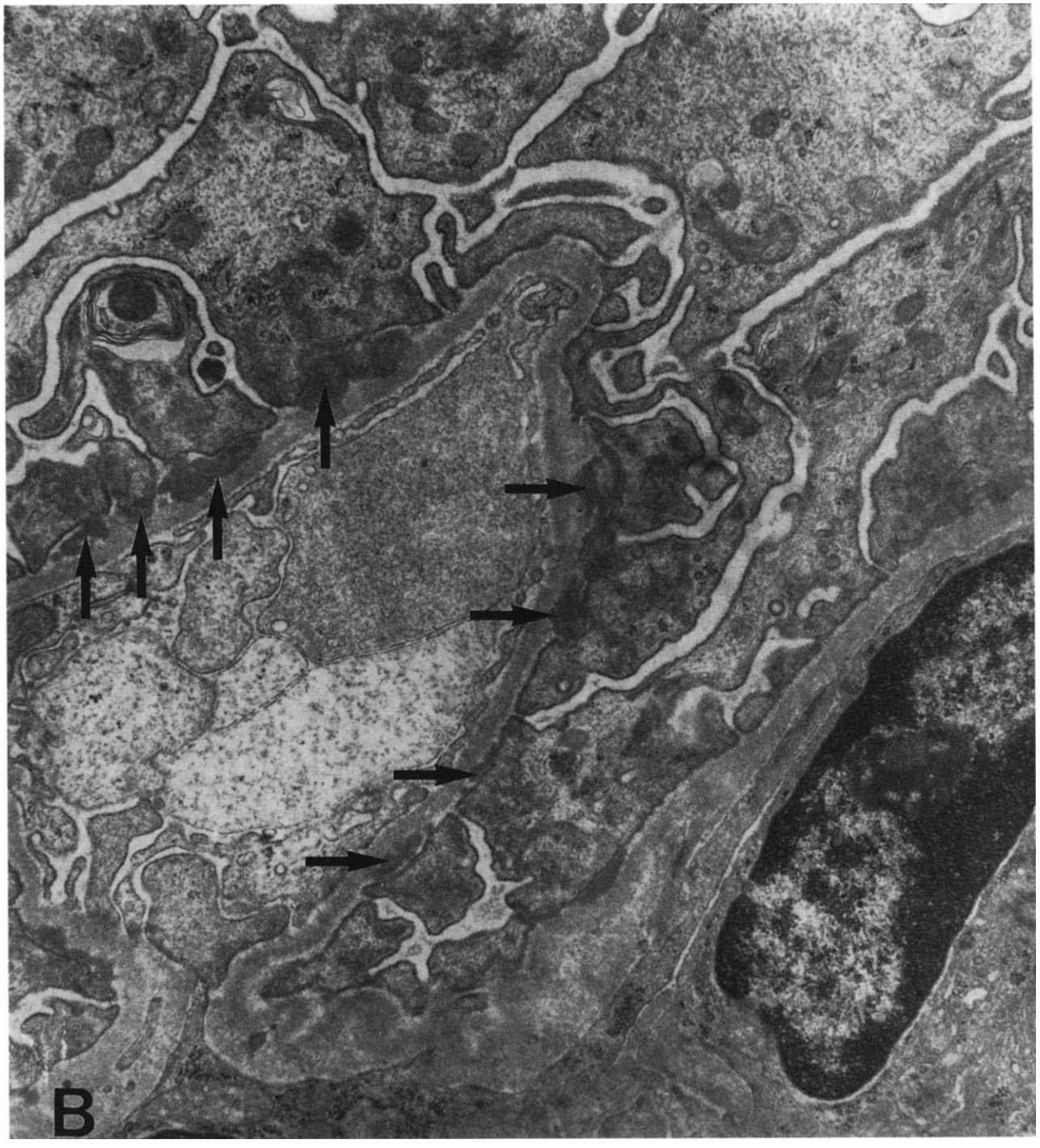

Fig. 6. *Continued.*

the finding of C5b-9 complex in electron dense deposits in biopsies of AHN [12]. The chance discovery of a C6 deficient rat provided an opportunity to examine whether the C5b-9 MAC was essential for the induction of glomerular injury and its resultant proteinuria in AHN. In this study we compared PVG rats deficient in the C6 component of complement to PVG rats with normal complement components which were immunologically identical. As would be expected the immune response to the immunization with Fx1A was identical in both groups of rats. High titers of autoantibodies were observed by three weeks post-immunisation, and there was evidence of equal deposition of autoantibody and C3 in the glomeruli of complement deficient and normal rats at four weeks post-immunization. Surprisingly there was no difference in the time course and the severity of the proteinuria in both groups of rats. This suggests that the MAC is not essential in inducing the glomerular injury which causes proteinuria in AHN. To examine whether there was a small difference in the onset of proteinuria or whether repletion of the

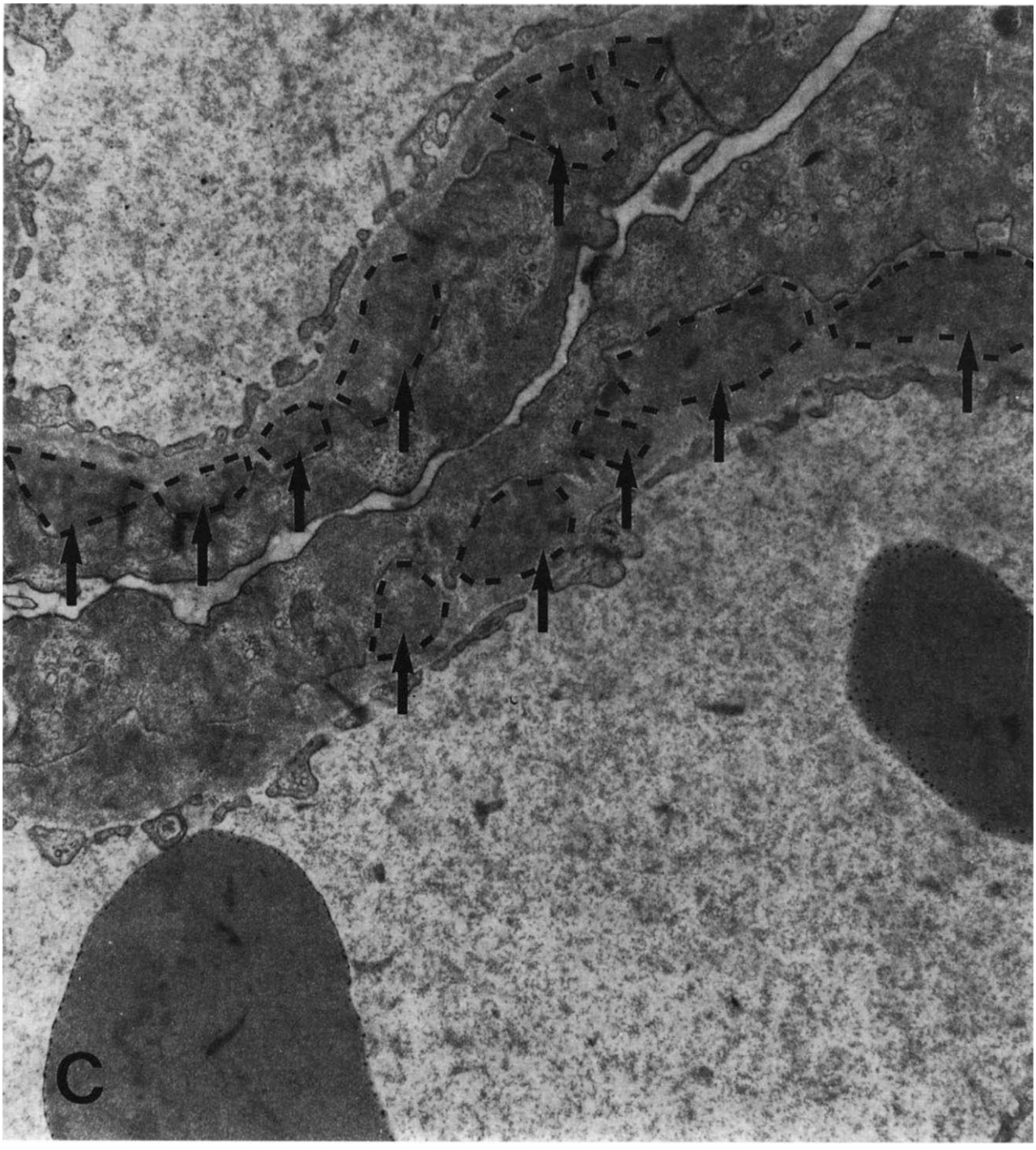

Fig. 6. *Continued.*

complement cascade would accelerate the disease, we undertook experiments where rats were reconstituted with rabbit serum at three weeks post-immunization. At this time they had high anti-Fx1A titers and Ig and C3 in their glomeruli. Although the complement cascade was partially restored as measured by the CH50 assay the evolution of the disease was not changed at all.

Taken together, these findings demonstrate that the C5b-9 complex does not play a role in AHN. The role of complement in models of nephritis has for technical reasons only been studied in acute models. This is due to the lack of rodents with established complement deficiencies and because depletion of complement factors is limited in time. Therefore, studies identifying a role for complement have used the passive form of HN [9–11]. In this model a heterologous phase followed by an autologous phase results in proteinuria and urinary C5b-9 loss within a few days after the administration of the heterologous antibodies [22]. While in PHN the whole process of glomerular membrane damage occurs within ten days, in the AHN model it takes at least

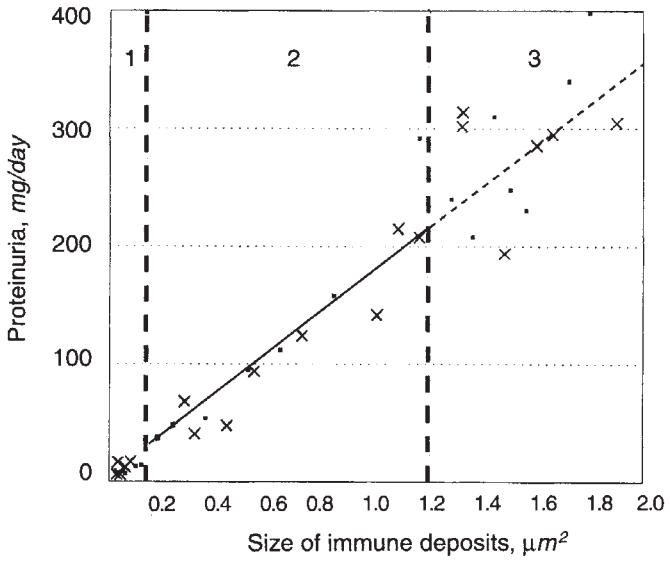

Fig. 7. *Relationship between size of the largest immune deposits of randomly chosen capillary loops and proteinuria.* Small deposits (**area 1**), observed at four weeks after induction of the disease, were not associated with significant proteinuria. A linear correlation ($r = 0.94$) was found from a size of 0.16 to 0.20 to 1.20 μm^2 (**area 2**). In nearly all animals these data points were obtained from biopsies taken between six and twelve weeks after disease induction. Later biopsies (**area 3**) showed large deposits of 1.30 to 2.00 μm^2 with a lesser correlation with proteinuria. Squares represent PVG/c$^-$ rats, crosses PVG/c$^+$ rats.

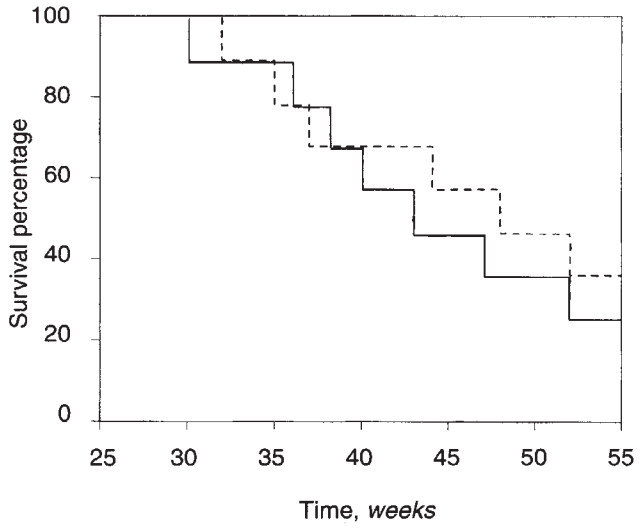

Fig. 8. *Long-term survival curves of ten PVG/c$^+$ (--) and PVG/c$^-$ (—) rats.* All animals showed severe proteinuria (range from 148 mg to 570 mg per day) which did not change substantially within the follow-up period.

six weeks before the development of proteinuria. This is despite the fact that in AHN high titers of autoantibody develop within three weeks of immunization and intraglomerular Ig, C3 and electron dense deposits are observed by four weeks. Why the C5b-9 complex only takes a few days to damage the glomerular membrane in PHN and several weeks in AHN has never been explained. That the C5b-9 complex is not critical for glomerular injury is not surprising since there is increasing evidence that this pathway may not be that relevant to the mediation of damage to nucleated cells *in vivo*. Not only are nucleated cells extremely resistant to complement damage [23, 24], they are also protected against homologous complement by membrane bound factors as CD59, decay accelerating factor (DAF) and others [21]. These factors tend to be specific in deactivating homologous complement. They are less resistant to heterologous complement. Nevertheless, our experiments demonstrated that even heterologous complement had no influence.

Our findings do not completely rule out a role of complement, however. The deficient rats showed early deposition of C3 which could not be distinguished from the amount of C3 found in the glomeruli of normal rats. It is not excluded that complement by release of chemotactic factors in both groups of animals may have contributed to glomerular injury [25]. Other studies have also demonstrated that antibody without complement activation can mediate glomerular injury. These include the infusion of monoclonal antibodies specific to glomerular antigens which do not fix complement but can induce glomerular injury and proteinuria [26, 27]. Further, in anti-GBM nephritis in guinea pigs decomplementation does not prevent glomerular injury [28]. In humans, individuals with complement pathway defects are prone to nephritis and not protected from it [29].

If the C5b-9 complex does not play the key role in AHN we wondered which other mechanisms cause the renal injury. We demonstrated that deposition of immunoglobulin and activation of the early components of complement did not immediately result in proteinuria. We therefore examined whether the form or size of the immune complexes were related to the onset of proteinuria. Earlier reports on other forms of experimental glomerulonephritis have focused on ultrastructural alterations during disease induction [30, 31]. Changes in the integrity of the slit pore membrane and detachment of glomerular epithelial cells seems to be related to the appearance or increase of proteinuria. Other parameters such as detachment, distortion of the slit pore membrane, localization of the deposit, length and size of the deposit and others may all influence the integrity of the glomerular membrane. A study of all these parameters and their contribution to glomerular membrane damage would require a large amount of EM biopsies and computerized planimetry. We limited our study to measuring the largest deposits in the two-dimensional plane of the EM photograph. It was found that deposits gradually increase in size and that simultaneously proteinuria appeared. Small deposits did not cause significant proteinuria. It seems that a minimal size is necessary to cause proteinuria. Further increase results in concomitant increase in proteinuria. Immune deposits reach their maximal size approximately twelve weeks after disease induction and remain virtually unchanged during the life span of the rats. The same is true for proteinuria. When immune deposits are relatively small, some form of slow endocytosis occurs, as has been shown by others [32–34]. This is in contrast to immune complexes at the capillary side of the basal membrane which are rapidly cleared [35]. Once a maximal size of the deposits has been reached, an equilibrium between growth and clearance exists or, and essentially not different, circulating autoantibodies hardly find nephritogenic antigens when large deposits are formed.

These data lead us to a picture of AHN of a true autoimmune disease with a slow progressive growth of immune deposits and a concomitant appearance of proteinuria. In contrast to models with monoclonal antibodies [26, 27, 36] AHN is not a model of

glomerulonephritis in which apparently crucial membrane structures are damaged which result in immediate proteinuria. Thus, the logical conclusion of the above is that a pure mechanistic theory explains the renal injury in AHN. Immune deposits damage the glomerular membrane and cause proteinuria by virtue of their size. Secondary mechanisms such as flattening and detachment of glomerular epithelial cells and distortion of slit pore membranes are the result of the growth of the immune deposits. These mechanisms are probably much more relevant for the proteinuria than size of the deposits on itself. More recently, similar data have been reported in human membranous nephropathy [37]. Insofar as AHN reflects human membranous nephropathy, these ideas might have important implications for its treatment.

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